Molecular & Biochemical Parasitology 221 (2018) 32–35

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

journal homepage: www.elsevier.com/locate/molbiopara

Heterologous expression of three antigenic proteins from *Angiostrongylus cantonensis*: ES-7, Lec-5, and 14-3-3 in mammalian cells

Bianca B. Cognato[a], Sukwan Handali[b], Alessandra L. Morassutti[a,*], Alexandre Januário da Silva[c], Carlos Graeff-Teixeira[a]

[a] *Laboratorio de Parasitologia Molecular, Instituto de Pesquisas Biomédicas and Laboratorio de Biologia Parasitaria, Faculdade de Biociências da Pontifícia Universidade Catolica do Rio Grande do Sul (PUCRS), Av Ipiranga 6690, 90690-900, Porto Alegre, RS, Brazil*

[b] *Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention, 1600 Clifton Road, MS D-64, Bldg 23, Room 9-440, Atlanta, GA, 30329, USA*

[c] *U.S. Food and Drug Administration, Center for Foods Safety and Applied Nutrition, Office of Applied Research and Safety Assessment, Division of Food and Environmental Microbiology, USA*

ARTICLE INFO

Keywords:
Angiostrongylus cantonensis
Recombinant proteins
Chinese hamster ovary cells
Human embryonic kidney cells
Glycoprotein

ABSTRACT

Angiostrongylus cantonensis is a parasitic nematode and the main causative agent of human cerebral eosinophilic meningoencephalitis (EoM). A definitive diagnosis of EoM usually requires serologic or molecular analysis of the patient's clinical sample. Currently, a 31 kDa antigen is used in immunological tests for this purpose, however as a crude antigen preparation it may present cross-reactivity with other helminthic infections, especially echinococcosis. Heterologous expression studies using prokaryotic systems failed on producing antigenic proteins. The aim of this study was to express and purify three recombinant glycoproteins representing *A. cantonensis* antigens: ES-7, Lec-5, and 14-3-3, in Chinese hamster ovary (CHO) cells and ES-7 in human embryonic kidney (HEK) cells to develop a source of specific antigens to be used in the diagnosis of angiostrongyliasis. The potential diagnostic value of these three proteins was subsequently characterized in one- and two-dimensional electrophoresis and Western blot to dot blot analyses, with *Angiostrongylus*-positive sera, normal human sera (NHS), and a pool of *Echinococcus*-positive sera (included as a specificity control) used for detection. In addition, recognition of these three proteins following treatment with *N*-glycosidase F was examined. The ES-7 proteins that were expressed in HEK and CHO cells, and the Lec-5 protein that was expressed in CHO cells, were specifically recognized by *A. cantonensis*-positive sera in the 2D electrophoresis analysis. This recognition was shown to be dependent on the presence of glycidic portions, making mammalian cells a very promising source of heterologous expression antigenic proteins from *Angiostrongylus*.

1. Introduction

Angiostrongylus cantonensis is the most common causative agent of EoM. Humans are an incidental host and they become infected following the ingestion of raw or undercooked snails or slugs. Paratenic hosts, such as prawns or contaminated vegetables, serve as hosts for infective larvae (L3). There have been many cases reported worldwide, especially in Asia and the Pacific Islands [1], but it has expanded worldwide [2–5].

A parasitological diagnosis of *A. cantonensis* is challenging since larvae are rarely detected in cerebrospinal fluid. Consequently, indirect methods are used for diagnosis. An antigen that has been used as a reliable immunodiagnostic for angiostrongyliasis corresponds to a 31 kDa protein band from whole crude antigen from female worms [6]. Previous work have identified and characterized several protein components present in this 31 kDa band using two-dimensional (2D) electrophoresis and demonstrated the that glycidic moieties in such proteins were immunogenic [7].

With the purpose of developing a more accurate source of antigen for diagnosis of human angiostrongyliasis, Morassutti and collaborators [8] expressed in prokaryotic cells the 14-3-3 protein, one of the components identified in the 31 kDa band, as well as the excretion and secretion products, Lec-5 and ES-7, also found as promising diagnostic targets in another study [9]. All three recombinant proteins were indiscriminately recognized by normal human sera (NHS). Based on these results, it was hypothesized that the incorporation of carbohydrates during protein expression may be crucial for protein antigenicity. It was also recognized that protein folding in a prokaryotic expression system

* Corresponding author at: Laboratorio de Biologia Parasitaria da Pontifícia Universidade Catolica do Rio Grande do Sul, Avenida Ipiranga 6690, Predio 12, Bloc°C, Sala 241, 90690 900, Porto Alegre, RS, Brazil.
E-mail address: almorassutti@gmail.com (A.L. Morassutti).

https://doi.org/10.1016/j.molbiopara.2018.03.001
Received 14 December 2017; Received in revised form 13 March 2018; Accepted 15 March 2018
Available online 16 March 2018
0166-6851/

may differ from the protein folding process in a eukaryotic organism, since important organelles for protein synthesis are lacking in prokaryotes [10].

The aim of this study was to express three diagnostic target proteins from *A. cantonensis,* ES-7, Lec-5, and 14-3-3, in two widely used mammalian expression systems, Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK) cells [11]. A preliminary evaluation for sensitivity and specificity of these antigens was done using sera samples from individuals diagnosed with angiostrongyliasis, Echinococcosis and NHS.

2. Materials and methods

2.1. Recombinant proteins

Cloning of recombinant proteins was performed by GenScript (Piscataway, NJ, USA). All three proteins were cloned into a pUC57 plasmid as fusion proteins with glutathione S-transferase (GST).

Expression and purification of the recombinant proteins were performed according to the following protocol. Briefly, 100 ml suspensions of CHO cells (approximately 2×10^7 cells/ml) in serum-free FreeStyle™ TM CHO Expression Medium (Life Technologies, Carlsbad, CA, USA) and HEK 293-6E cells (approximately 2×10^7 cells/ml) in serum-free FreeStyle™ 293 Expression Medium (Invitrogen) were grown in Erlenmeyer flasks on an orbital shaker at 37 °C under 5% CO_2. Twenty-four hours later, DNA and polyethylenimine (PEI) (Polysciences, Eppelheim, Germany) were mixed at an optimal ratio of 5:1 (PEI:DNA) and then were added into each flask. Recombinant plasmids encoding ES-7, 14-3-3, and Lec-5 were each transfected into the CHO cell cultures. In parallel, the recombinant plasmid encoding ES-7 was transfected into a 100 ml suspension of HEK 293-6E cells, since ES-7 was previously shown to have great potential as a diagnostic target (Morassutti et al. [7]). After 6 days, all of the cell culture supernatants were centrifuged, filtered, and added to Glutathione Sepharose 4 HP resin columns (5 ml for CHO supernatants and 3 ml for HEK-6E supernatants, GE Healthcare, Uppsala, Sweden, Cat. no. 17-5279-01) at 1 ml/min. After approximately 4 h, the resins were washed and then eluted with an appropriate buffer. The purified proteins were then analyzed by SDS-PAGE and Western blotting according to standard protocols and molecular weights, yields, and purities, were recorded. Protein quantification was obtained by Thermo Scientific™ NanoDrop™ UV–vis spectrophotometers. To confirm the recombinant expression, a conjugated anti-GST-horseradish peroxidase primary antibody was used for Western blot analyses (GenScript, Cat. no. A00866). Moreover, the identities of all three proteins were confirmed by mass spectrometry analysis.

2.2. Serum samples

To test the antigenicity of the purified recombinant proteins, Western blot analyses were performed with *Angiostrongylus*-positive sera (which included sera from 20 individuals and a pool of 5 selected individual positive sera, normal human sera (NHS), which was pooled from 20 individuals that had no exposure to *A. cantonensis* and a pool of *Echinococcus granulosus*-positive sera, as a specificity control. All of the sera were diluted 1:100 when used. The serums obtained from *Angiostrongylus*-infected patients originated from biobanks from Pontifical Catholic University of Rio Grande do Sul (PUCRS). The use of these sera for this study was approved by the ethics committee of our institution. In addition, the specificity controls used were obtained from the serum bank from the Immunochemistry Laboratory of the Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention (CDC).

2.3. SDS-PAGE electrophoresis (1D electrophoresis)

All recombinant proteins were subjected to 1D electrophoresis with Mini-PROTEAN® TGX™ Precast Gels (15%, Bio-Rad Laboratories). Sample buffer [10% SDS, 6% glycerol, and tracking dye (50 mg bromophenol blue, 8 ml glycerol, 1 ml 0.5 M Tris HCL (pH 8.0), 1 ml distilled water)] with or without 100 mM dithiothreitol (DTT). Protein separation was achieved at a voltage of 75 V for 25 min with the Bio-Rad MiniProtean electrophoresis system. The amount of protein separated with 1D electrophoresis included: 0.12 ug of ES-7 produced in HEK cells, 0.15 ug of ES-7 produced in CHO cells, 0.07 ug of Lec-5, and 0.1 ug of 14-3-3. Detection of IgG4 was performed as a loading control.

2.4. Two-dimensional (2D) electrophoresis

Each recombinant protein was desalted with a 2-D Clean-Up Kit (GE Healthcare, Piscataway, NJ, USA) and then subjected to solubilization with DeStreak Rehydration Solution (GE Healthcare) containing 66 mM DTT (Sigma-Aldrich, Canada) and 0.5% carrier ampholytes. The samples were in-gel rehydrated on 11 cm pH 3–11 NL IPG strips (GE Healthcare) overnight and then isofocalized with the following stepwise increases in voltage: 500 V for 500 V h, a linear gradient from 500 to 8000 V for 6500 V h, followed by a hold at 6000 V for 22,000 V h. After that, the strips were soaked for 15 min in fresh equilibration buffer (20% v/v glycerol, 6 M urea, 1% DTT, and 2% SDS). IPG strips were run in the second dimension on Criterion XT Precast Gels 4–12% Bis-Tris IPG (Bio-Rad Laboratories) and then were stained with colloidal Coomassie blue or transferred to nitrocellulose membranes for the immunological assay. The amount of protein separated with 2D electrophoresis included: 20 ug of ES-7 produced in HEK cells, 0.5 ug of ES-7 produced in CHO cells, 14 ug of Lec-5, and 1.1 ug of 14-3-3. Detection of IgG4 was performed as a loading control.

2.5. Western blot analysis

Resolved proteins were transferred onto nitrocellulose membranes with a semi-dry trans-blot apparatus (Bio-Rad) and tested for specificity against *Angiostrongylus*-positive sera, NHS, and a specificity control. The membranes were blocked with 5% skim milk for 1 h at room temperature and then were incubated for 2 h with a pool of positive or negative serum or a specificity control diluted at 1:100. After three washes with PBS Tween (0.05%), the membranes were incubated with a secondary peroxidase-conjugated anti-human IgG antibody diluted at 1:8000 and an IgG4 antibody diluted at 1:1000 for 1 h at room temperature. Bands were visualized with after the strips were incubated with 0.05% diaminobenzidine (DAB; Sigma-Aldrich), 0.015% H_2O_2, in PBS, pH 7.4.

2.6. N-glycosidase F (PNGase F) treatment for deglycosylation

PNGase F treatment was performed according to the manufacturer's instructions (5×10^5 U/mL; BioLabs, United Kingdom). To investigate the antigenicity of the proteins in the absence of carbohydrates, PNGase F was mixed with each of the four recombinant proteins (Lec-5, 14-3-3, ES-7-HEK, and ES-7-CHO and PBS as a control) at the concentrations listed in Section 2.4. Then, each of the samples were incubated overnight at 37 °C with 500 U of PNGase F. Recognition of the carbohydrate moieties by positive and negative sera was tested by dot blot.

2.7. Dot blot

Recombinant proteins were blotted onto nitrocellulose membranes at concentrations of 1.4 ug, 5.5 ug, 0.5 ug, and 2.5 ug for Lec-5, 14-3-3, ES-7-HEK, and ES-7-CHO, respectively. Each membrane was treated as described for Western blot analysis.